Changes in Conformation of Insolubilized Trypsin and Chymotrypsin, Followed by Fluorescence*

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ABSTRACT: A fluorometric technique is described for the study of conformational changes in proteins covalently bound to insoluble carriers. The fluorescence peak of chymotrypsin bound to cyanogen bromide activated Sephadex or agarose shifted to the red in the presence of 8 M urea, similarly to chymotrypsin in solution. The insoluble chymotrypsin derivatives as well as chymotrypsin lost all of their enzymic activity under these conditions. A similar behavior as regards fluorescence and enzymic activity was exhibited by trypsin bound to agarose as well as trypsin in solution. Trypsin bound to Sephadex, on the other hand, which retains most of its activity in 8 M urea, yielded a fluorescence spectrum similar to that of the native enzyme. The chymotrypsin-Sephadex conjugate was fluorometrically found to bind 2-*p*-toluidinylnaphthalene-6-sulfonic acid (TNS) with the same binding constant as the native enzyme. 3-Phenylpropionic acid was found to bind to the chymotrypsin-Sephadex–TNS complex with the same binding constant as to the native chymotrypsin–TNS complex. The extent of quenching of the fluorescence of the bound TNS by 3-phenylpropionic acid was, however, considerably smaller in the insoluble enzyme than in the native one. It was thus suggested that whereas the Sephadex matrix did not appreciably modify the binding sites for TNS or substrate, it did interfere with the interaction between these sites. The chymotrypsin-Sephadex conjugate, heat treated for 15 min at 60° and cooled to 25°, showed the fluorescence properties of heat-denatured chymotrypsin in solution, but exhibited appreciable catalytic activity.

Biologically active proteins, such as enzymes and antibodies, artificially bound to insoluble carriers, are of theoretical and practical interest (Silman and Katchalski, 1966; Goldstein and Katchalski, 1968; Kay, 1968). Protein-carrier conjugates may serve as model systems for proteins embedded in biological membranes or other native complex structures. They can also be used in affinity chromatography and as specific heterogeneous catalysts.

The analysis of the characteristic catalytic behavior of immobilized enzymes carried out so far was based on the assumption that the observed differences in kinetics of immobilized enzymes and the corresponding native enzymes in solution are due to the modification by the carrier of the microenvironment in which the enzymes act. The effect on the apparent catalytic activity of the electrostatic potential induced by the matrix, restrictions due to diffusion of substrate and product, and exclusion of high molecular weight substrates have been discussed extensively (Goldstein *et al.*, 1964, 1967; Goldman *et al.*, 1968, 1971; Hornby *et al.*, 1968; Wilson *et al.*, 1968; Axén *et al.*, 1970; Cresswell and Sanderson, 1970). Tacitly it has been assumed that the conformation, and thus the intrinsic catalytic activity, of the immobilized enzymes is identical with that of the corresponding native enzymes. It should be borne in mind, however, that this might not always be the case. As a matter of fact, the differences recorded in stability to inactivation by heat or denaturing agents of bound enzymes as compared with the corresponding native enzymes (Wilson *et al.*, 1968; Erlanger *et al.*, 1970; Gabel *et al.*, 1970; Stasiw *et al.*, 1970; Surovtzev *et al.*, 1970; Glassmeyer and Ogle, 1971; Tosa *et al.*, 1971) suggest that changes in conformation or alterations in the ease of conformational change have resulted from the binding of the enzymes to the carriers.

Among the various physical–chemical methods developed to study protein conformation in solution, fluorescence techniques seem to be the most readily adapted to conformational studies of insolubilized proteins. The intensity as well as the spectrum of the emitted fluorescence light depends on the environment of the fluorescent groups. Changes in the environment are reflected in corresponding changes in the intensity and spectrum of the light emitted (Gally and Edelman, 1964; Steiner *et al.*, 1964). The strong scattering of light by the binding matrix might cause grave difficulties in the measurement of the fluorescence light. Fortunately, however, the protein concentration in insolubilized enzymes, and therefore the optical density at the protein absorption bands, is generally high. Light absorption thus competes effectively with light scattering. The exciting light is absorbed within a very thin layer at the face of a bed of protein–matrix conjugate. The fluorescence should therefore be collected from the front face of the bed. Furthermore, since the emitted light is of longer wavelength than the exciting light, fluorescence light can be readily separated from the scattered light.

In the following we describe a cell which allows the study of the fluorescence of insolubilized enzyme. The cell can be used in most commercial and self-built spectrofluorometers. With the aid of this cell, it was possible to investigate fluorometrically conformational changes in trypsin and chymotrypsin covalently bound to agarose or Sephadex, caused by urea, heat, and specific ligands. In some of the cases it was found that the changes in fluorescence paralleled the activity changes, whereas in other cases no such parallelism was observed and the fluorescence data yielded information concerning conformational changes of the insolubilized enzymes which could not be detected by activity measurements.

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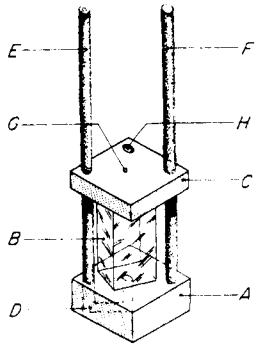

FIGURE 1: Cell and cell holder for front-face fluorimetry. (A) Bottom of the cell holder (PTFE, 3 mm thick); (B) microcell (quartz); (C) top of the cell holder (PTFE, 2 mm thick); (D) channel leading from the inner of the cell to tube E; (E) tube of stainless steel serving as drainage; (F) tube of stainless steel, serving with tube E as support; (G) hole to insert a hypodermic needle into the cell; and (H) boring to accommodate a thermistor.

Materials and Methods

Trypsin and chymotrypsin were purchased from Worthington; β-alanyl-L-tryptophan was a gift from Dr. M. Wilchek; 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK)[1] was purchased from E. Merck and Co.; Dextran T-500, Sephadex G-200, and agarose in the form of Sepharose 2B were products of Pharmacia Fine Chemicals. Urea (AnalaR, BDH) was recrystallized from 70% ethanol before use. 2-*p*-Toluidinylnaphthalene-6-sulfonic acid (TNS) (Eastman Kodak) was recrystallized from methanol–water. All other reagents were of analytical grade, and were used without further purification.

Trypsin, chymotrypsin, and β-alanyl-L-tryptophan were coupled to cyanogen bromide activated Sephadex G-200 (activation at pH 10.0) or Sepharose 2B (activation at pH 11.0), as described previously (Axén *et al.*, 1967; Axén and Ernback, 1971). TLCK-trypsin was prepared as described elsewhere (Shaw *et al.*, 1965).

pH Measurements. pH values were measured with a Radiometer pH meter PHM 28 and a combination glass calomel electrode at 25°. No corrections were made for the effect of solvent on the response of the electrode to hydronium ion activity in aqueous 8 M urea solution.

Determination of Enzymic Activity. The esterolytic activity of chymotrypsin was determined with a pH-Stat (Radiometer) under helium, using *N*-Ac-L-TyrOEt (0.01 M in 0.3 M KCl–5% ethanol) as substrate. The measurements were carried out at the apparent pH optima (pH 7.9 for the native enzyme and pH 9.7 for the chymotrypsin-Sephadex conjugate). The temperature was maintained constant by circulating water from a thermostat through the jacketed cell holder. Blank experiments were performed to determine alkali uptake in the absence of enzyme. The values of K_M and V_{max} for the bound chymotrypsin at 25° were derived from Lineweaver–Burk plots, using *N*-Ac-L-TyrOEt as substrate at the concentration range of 5–15 mM.

Activity of Chymotrypsin and Chymotrypsin-Sephadex at Different Temperatures. Chymotrypsin (*ca.* 10 μg in 10 μl) was added to 1.9 ml of 0.32 M KCl solution (pH 7.2), preheated to the desired temperature. After incubation for 5 min at the temperature specified, 0.2 M *N*-Ac-L-TyrOEt in 99% ethanol (0.1 ml) was added and the enzymic activity determined pH-statically. A similar procedure was adopted for the determinations of the enzymic activity of chymotrypsin-Sephadex at different temperatures, except that incubation in the salt solution before adding the substrate was prolonged to 15 min.

Heat Inactivation of Chymotrypsin and Chymotrypsin-Sephadex. Chymotrypsin (*ca.* 10 μg in 10 μl) was added to 1.9 ml of 0.32 M KCl solution (pH 7.2), preheated to the desired temperature. After incubation for 5 min at the temperature specified the mixture was cooled to 25°. Fifteen minutes later 0.2 M *N*-Ac-L-TyrOEt in 99% ethanol (0.1 ml) was added and the enzymic activity determined pH-statically. A similar procedure was adopted for the determination of heat inactivation of chymotrypsin-Sephadex, except that incubation in the 0.32 M KCl solution prior to cooling was prolonged to 15 min.

Flow Cell for Front-Face Fluorimetry. A special cell was designed for the study of the fluorescence of the enzyme–carrier complexes under various conditions. It consisted of a micro cell (quartz, 5.4 mm outer width, 3.0 mm inner width, obtained from American Instrument Co., catalog no. 4-8114) the bottom of which was cut off, and an appropriate holder for this cell, fitting into an ordinary 1.0 × 1.0 cm fluorescence cell. The cell holder consisted of two square pieces of polytetrafluorethylene (PTFE) (A and C) and two tubes of stainless steel (E and F) (see Figure 1). Into the PTFE piece serving as a bottom (A) a groove was carved into which the walls of the micro cell fitted. The angle between the front face of the cell and the exciting beam was 30° in the Aminco-Keirs spectrofluorometer and 60° in the Turner spectrofluorometer. The center of the outer cell was inside the space enclosed by the micro cell and 0.3 mm from its inner front face. (A geometrically similar arrangement for the measurement of surface fluorescence of solid samples has been proposed by Chen *et al.* (1969).) A hole was drilled in the bottom plate at the center of the space enclosed by the inner cell and filled with a fitting piece of porous plastic (Bel Art). The hole led to one of the stainless steel tubes (E) *via* a boring (D). The cover (C) was made similar to the bottom and a hypodermic needle was passed through it to allow change of the liquid inside the cell. It was found necessary to surround the upper and lower ends of the cell with a water-resistant glue (Ubu, Fischer-Werke), which does not release uv-absorbing or fluorescent substances, in order to avoid leakage. A hole (H) was bored through the cover so that a thermistor could be inserted into the outer cell.

The inner cell was packed with the enzyme-carrier gel by means of a Pasteur pipet. After the glue dried the cell holder was inserted into the outer cell, which was filled with 1 ml of water. When necessary, the solution inside the micro cell was replaced by forcing new liquid through the cell from its top. A few centimeters of hydrostatic pressure were usually applied.

It is pertinent to note that identical fluorescence spectra were obtained for β-alanyl-L-tryptophan, trypsin, and chymotrypsin when measured with the micro cell described or with a conventional 1.0 × 1.0 cm cell.

Spectrofluorometers. The following spectrofluorometers were used: a Turner Spectro 210 (G. K. Turner Associates) and an Aminco-Keirs spectrofluorometer (American Instrument Co.) equipped with a thermostated cuvet holder. In the

[1] Abbreviations used are: TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone; TLCK-trypsin, trypsin inactivated by TLCK; β-alanyl-tryptophan-Sephadex, chymotrypsin-Sephadex, chymotrypsin-agarose, trypsin-Sephadex, and trypsinagarose, conjugates of the peptide or the enzymes obtained by their coupling with cyanogen bromide activated Sephadex or agarose; TNS, 2-*p*-toluidinylnaphthalene-6-sulfonic acid; PTFE, polytetrafluoroethylene.

latter apparatus, a RCA phototube type 1P28 was used for measurements in the uv and type 1P21 for measurements in the visible part of the spectrum. The Turner apparatus gives energy-corrected spectra, whereas the spectra obtained from the Aminco apparatus were not corrected and thus differed to some extent from those obtained with the former instrument. As the gel which fills the micro cell prevents the exciting beam from passing through, the built-in mechanism for compensation in light source fluctuations in the Turner will not operate. Therefore the mode selector switch of this apparatus was set to "luminescence" mode, at which the spectra are corrected for the differences in spectral response of the detector system only. The spectral bandwidth of the excitation and fluorescence light was 10 nm. It was necessary to cover the lower part of the cuvet facing the exciting beam with black tape in order to diminish scattering due to the glue surrounding the bottom of the inner cell. When the Aminco-Keirs spectrofluorometer was used, this was found unnecessary when the original cell support was replaced by a thinner piece of plastic or brass and suitable slits were inserted into the excitation beam.

Fluorescence Measurements. The fluorescence of β-alanyltryptophan was measured in 0.1 M sodium phosphate buffer (pH 6.0), containing 8 M urea when indicated. In the pH range 7–11, 0.1 M sodium borate buffers were used. The concentration of the peptide was *ca.* 0.2 mg/ml.

The fluorescence of the proteins was measured throughout in 0.01 M sodium borate buffer (pH 8.1) (as determined at 25°) containing 0.1 M sodium chloride. The borate buffer shows relatively little change in pH with temperature. For the fluorescence measurements in the presence of urea, the appropriate urea solution was passed through the gel and the spectrum recorded after 20 min. Thereafter the gel was washed with buffer and the fluorescence measured again after 20 min. For the study of the effect of temperature on the fluorescence of bound chymotrypsin, the sample investigated was put in the micro cell which was then inserted into the thermostated cell holder. The spectrum was recorded 5 min after a constant temperature was reached (as monitored by the inserted thermistor). The fluorescence of chymotrypsin in solution was measured 5 min after adding 50 μg of enzyme in 0.1 ml of water to borate buffer (2 ml) heated to the desired temperature. The measurements in the presence of TNS and 3-phenylpropionic acid were performed similarly to those with urea. In all cases where the fluorescence of the enzyme gels was measured, the samples were exposed to the exciting beam only for the time required to record the fluorescence spectrum. By this procedure, photoreactions were minimized and the original gels could be repeatedly studied.

Unpolarized light was used throughout for excitation, and the fluorescence was measured at 90° to the excitation beam. It can be readily shown that under these conditions the total emitted light is proportional to $[(3 + p)/2]I_{90°}$, where $I_{90°}$ is the fluorescence intensity at 90° to the excitation beam, and p is the polarization of the fluorescence in this direction. For excitation by unpolarized light, the upper limit for p is $^1/_3$ and can assume the value of zero upon complete depolarization. Moreover, changes in p in any experiment are hardly expected to encompass the whole permissible range for p. It may thus be concluded that changes in $I_{90°}$ approximately reflect changes in total emitted light.

Results

Fluorescence of β-Alanyl-L-tryptophan. Sephadex or Sephadex activated with cyanogen bromide did not show any fluorescence when irradiated at 280 or 360 nm. No fluorescent impurities are thus present in any detectable amounts.

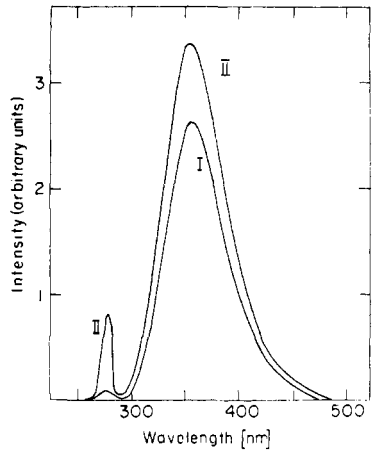

FIGURE 2: Fluorescence spectrum of free β-alanyltryptophan (curve I) and of β-alanyltryptophan bound to Sephadex (curve II), both measured in 0.1 M sodium phosphate buffer (pH 6.0). Measurements were carried out with an Aminco-Keirs spectrofluorometer. Wavelength of excitation, 280 nm. The two spectra are not on the same intensity scale.

The peptide β-alanyltryptophan at pH 6.0 emitted fluorescent light with a maximum at 355 nm upon irradiation at 280 nm (Figure 2, curve I). The same fluorescence spectrum was obtained with the micro cell as with a regular 1.0 × 1.0 cm cell. The presence of Dextran T-500 at a 10% concentration (pH 6.0) did not affect either the shape or the intensity of the spectrum. β-Ala-Trp covalently bound to Sephadex G-200 exhibited the same fluorescence spectrum, as seen in Figure 2, curve II. The peak of scattered light was well separated from the fluorescent light. Urea (8 M at pH 6.0) increased the intensity of the spectrum of both the free and the bound peptide by 50%; no shift in wavelength was, however, noted in either case. Upon washing with buffer the bound peptide regained its original fluorescence intensity. Prolonged exposures of the gel to the excitation beam (10 min or more) resulted in a loss in fluorescence intensity, probably as a result of photoreactions. The effect of pH on the fluorescence intensity of the free and the bound peptide was measured in 0.1 M sodium borate buffers. The fluorescence intensity of free peptide followed a titration curve corresponding to an ionization pK of 9.3, the intensity being 60% lower at pH 7 than at pH 11 (Figure 3), in agreement with previous reports (Edelhoch *et al.*, 1967). The fluorescence of the bound peptide was also quenched upon lowering the pH from 10 to 7, but the titration curve did not fit a single ionization constant, and the extent of quenching was only half of that observed for the free peptide (Figure 3). Below pH 7 no further quenching was observed down to pH 5.

The fluorescence spectrum of TLCK-trypsin was similar to that of trypsin (both measured at pH 8.1), the maxima being at 335 and 333 nm, respectively. When the micro cell was filled with swollen Sephadex G-75, the shape of the spectrum did not change, but there was a slight loss in intensity.

Influence of Urea on the Fluorescence of Trypsin, Chymotrypsin, and Their Insoluble Derivatives. The fluorescence spectra of agarose- and Sephadex-bound chymotrypsin and trypsin could be measured readily without interference from the peak of scattered light. All spectra resembled in shape those of the

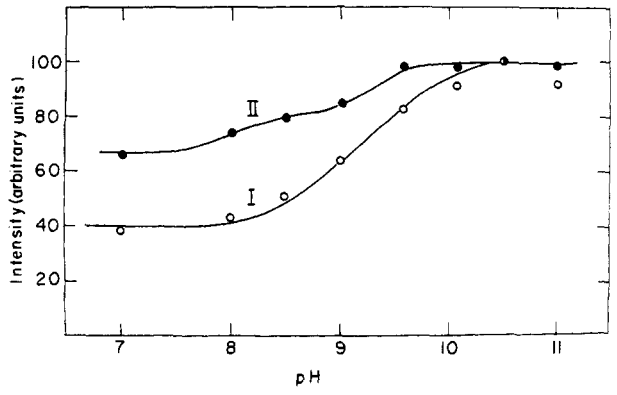

FIGURE 3: Variation of the fluorescence intensity with pH of free β-alanalytryptophan (curve I) and of β-alanyltryptophan bound to Sephadex (curve II). Fluorescence was measured with an Aminco-Keirs spectrofluorometer at the emission peak (355 nm). Excitation wavelength, 280 nm. The intensity at pH 10.5 was arbitrarily set to 100.

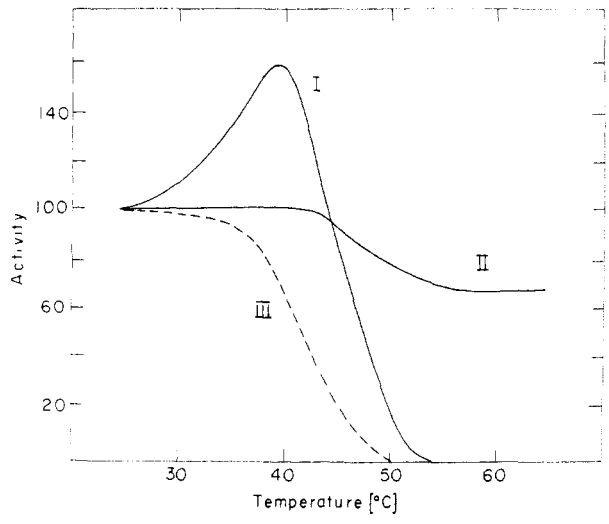

FIGURE 5: Enzymic activity and stability of chymotrypsin and of chymotrypsin-Sephadex at different temperatures. *N*-Ac-L-TyrOEt (0.01 M) in 0.3 M KCl was used as substrate throughout. Curve I: enzymic activity of chymotrypsin and of chymotrypsin-Sephadex measured at the temperatures specified. Curve II: residual enzymic activity of chymotrypsin-Sephadex, measured at 25°, after preheating for 15 min at the temperatures specified. Curve III: residual enzymic activity of chymotrypsin measured at 25°, after preheating for 5 min at the temperatures specified. For further details, see text.

free enzymes. Figure 4 presents the fluorescence spectrum of trypsin-Sephadex at pH 8.1, measured in a Turner spectrofluorometer. Urea affected the spectra of chymotrypsin-Sephadex, chymotrypsin-agarose, and trypsin-agarose in essentially the same way as it affected the spectra of the free enzymes. In all three cases there was a red shift of about 20 nm in the fluorescence peak in 8 M urea compared to water solution as measured in the Aminco-Keirs apparatus. Chymotrypsin-agarose showed a decrease in fluorescence intensity upon transfer to 8 M urea, in contrast to the increase in intensity observed for chymotrypsin in solution and chymotrypsin-Sephadex. This might be due to the observed increase in bed volume of the agarose derivative in 8 M urea. Upon washing the trypsin- and chymotrypsin-Sephadex gels with borate buffer (pH 8.1), the wavelength of maximum fluorescence did not return to the original value, but showed only a slight blue shift relative to its value in urea and a decrease in intensity. In connection with the above it is pertinent to note that trypsin and chymotrypsin in solution as well as trypsin-agarose, chymotrypsin-Sephadex, and chymotrypsin-agarose lose their enzymic activity in 8 M urea (Gabel *et al.*, 1970; P. Vretblad and D. Gabel, unpublished results).

In accord with previous findings that the enzymic activity of trypsin-Sephadex is retained in 8 M urea (Gabel *et al.*, 1970),

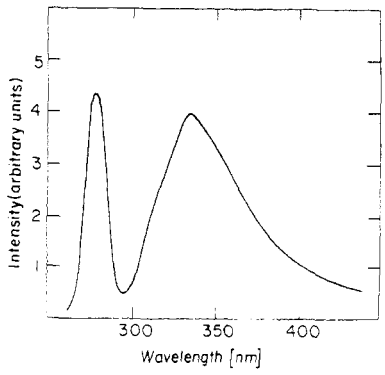

FIGURE 4: Fluorescence spectrum of trypsin-Sephadex at pH 8.1 and 25°, measured with a Turner spectrofluorometer. Excitation wavelength, 280 nm. Spectral band widths of the excitation and emission light, 10 nm.

only a small red shift in the fluorescence peak from 335 to 338 nm and no change in the intensity at the peak (as measured in the Turner spectrofluorometer) were found. Upon washing the bed with borate buffer (pH 8.1) the original spectrum, both as regards shape and intensity, was recovered.

Effect of Temperature on Activity and Fluorescence of Chymotrypsin and Chymotrypsin-Sephadex. Chymotrypsin shows maximal activity at temperatures around 38°. At higher temperatures, the activity drops rather quickly (Figure 6, curve I) and the enzyme does not recover its original activity upon cooling to 25° (Figure 5, curve III) (see also Surovtzev *et al.*, 1970).

In parallel experiments it was found that the fluorescence of chymotrypsin as a function of temperature followed a somewhat different course (Figure 6). No shift in the emission peak (335 nm) was observed at 38° where the activity starts to drop; instead, the peak started to shift to longer wavelengths at 47°, where the enzyme is already irreversibly inactivated, eventually reaching a value of 355 nm. (These data were obtained with the Aminco-Keirs spectrofluorometer and are not corrected for instrument response.) Similarly, the slope of the intensity (at the wavelength of maximum emission) *vs.* temperature changed at 47° (Figure 6). Upon cooling of chymotrypsin which had been denatured at 60° for 5 min (pH 8.1), the fluorescence intensity increased, but did not reach the original value, and the wavelength of maximum emission remained constant at 355 nm.

The change in activity of chymotrypsin-Sephadex with temperature followed the same curve as the free enzyme (Figure 5, curve I). The fluorescence decreased upon heating; however, the slope of intensity *vs.* temperature did not change at 48° (Figure 7). At this temperature, the wavelength of maximum emission did change upon further heating, starting from 335 nm, eventually reaching a value of 352 nm (uncorrected). Upon cooling from 63°, the insolubilized chymotrypsin showed the same behavior as the free enzyme; the

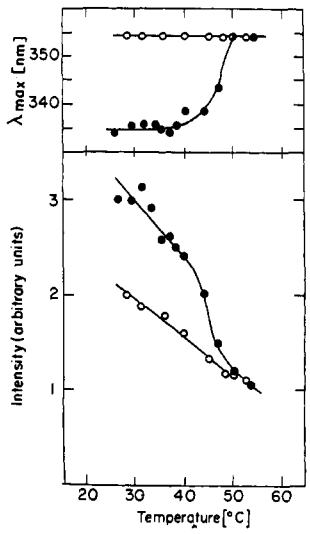

FIGURE 6: Effect of temperature on the tryptophan fluorescence of chymotrypsin at pH 8.1, as measured with an Aminco-Keirs spectrofluorometer. Excitation wavelength, 280 nm. (●) Values obtained on heating. (○) Values obtained on cooling after fast heating to 60°. The intensity values are those at the wavelength of maximum emission.

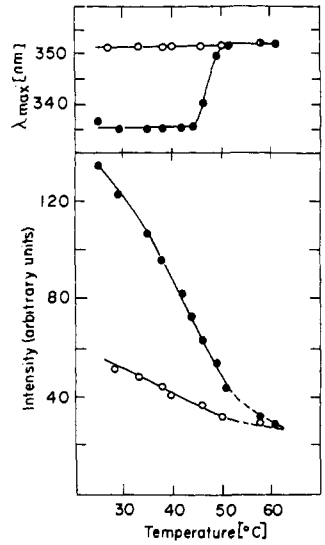

FIGURE 7: Effect of temperature on the tryptophan fluorescence of chymotrypsin-Sephadex at pH 8.1, as measured with an Aminco-Keirs spectrofluorometer. Excitation wavelength, 280 nm. (●) Values obtained on heating. (○) Values obtained on cooling from 63°. The intensity values are those at the wavelength of maximum emission.

fluorescence intensity increased but the emission peak remained at 352 nm.

Curve II of Figure 5 represents the stability of chymotrypsin-Sephadex to heat inactivation. The enzyme stability was monitored by the residual activity measured at 25° after exposure to higher temperatures for 15 min. The stability of the conjugate decreased at temperatures above 45°, but 70% of the initial activity remained even after exposure to 65° as judged by the assay procedure used (0.01 M *N*-Ac-L-TyrOEt). V_{max} for *N*-Ac-L-TyrOEt as substrate was found to drop to 40% of its original value after heating the insolubilized enzyme to 60°. K_M(app) dropped from 25 to 17 mM. It should be noted that due to diffusion of substrate into the gel, K_M(app) is not the true K_M of the enzyme and depends on V_{max} (T. Steinberg, D. Gabel, and E. Katchalski, to be published). Attempts to avoid the effect of diffusion by using *N*-acetyl-L-phenylalanine-*p*-nitroanilide as substrate were not successful because of a too slow hydrolysis by the heat-treated enzyme. Chymotrypsin-Sephadex samples kept at 65° for several hours irreversibly lost all activity as assayed at room temperature. Incubation at 45° up to 24 hr did not lead, however, to inactivation greater than that recorded after 15 min.

Binding of TNS to Chymotrypsin-Sephadex. Chymotrypsin-Sephadex bound TNS (2-*p*-toluidinylnaphthalene-6-sulfonic acid) similarly to the free enzyme. Upon binding to the insolubilized protein the dye became fluorescent, with an emission peak at 455 nm (excitation wavelength 360 nm). The dissociation constant for this reaction was determined by (McClure and Edelman, 1967)

$$I = I_{max} - K_{diss}\frac{I}{c} \quad (1)$$

where I is the measured intensity; I_{max}, the fluorescence of the solution when all enzyme molecules are saturated with dye; K_{diss}, the dissociation constant of the complex; and c, the concentration of free dye. When the concentration of enzyme is small compared to the dissociation constant, the concentration of the free dye, c, can be approximated by the concentration of total dye present, c_{total}. A plot of I *vs.* I/c_{total} should yield a straight line with slope K_{diss} (Figure 8). The measurements were done with a total dye concentration in the range 1.8×10^{-5} to 7×10^{-4} M. The approximate enzyme concentration in the chymotrypsin-Sephadex gel was estimated to be 4×10^{-5} M (see Discussion; in view of earlier reports (Axen *et al.*, 1970) the true value might be considerably lower). The dissociation constant thus obtained was 1.7×10^{-4} M, as compared to 2×10^{-4} M for the free enzyme. (The value obtained for K_{diss} justified the replacement of c by c_{total}.) As shown in Figure 8, curve II, the heat-treated chymotrypsin-Sephadex showed a different binding behavior toward TNS. The slope of the curve is steeper, and the intensity value

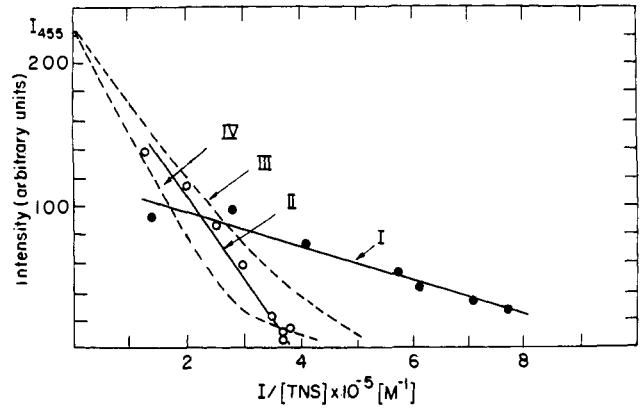

FIGURE 8: Fluorescence titration of chymotrypsin-Sephadex with TNS, plotted according to eq 1. All measurements were carried out at 26° and pH 8.1. Excitation wavelength, 360 nm. The fluorescence was recorded at 455 nm with an Aminco-Keirs spectrofluorometer. Curve I: chymotrypsin-Sephadex (not heated). Curve II: chymotrypsin-Sephadex after exposure to 60° for 15 min. Curves III and IV: calculated curves for mixtures of two species with different binding constants for TNS. For further details, see Discussion.

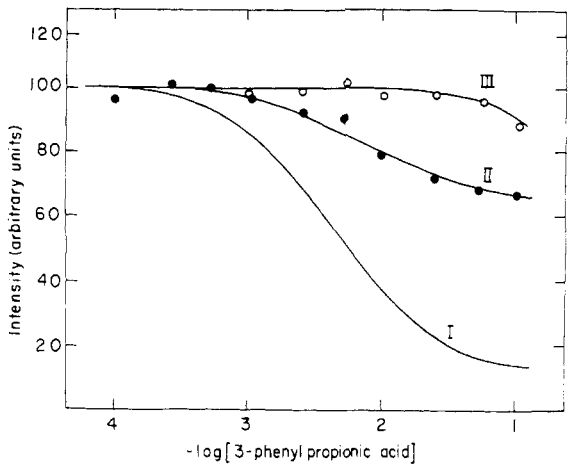

FIGURE 9: Quenching of the fluorescence of TNS bound to chymotrypsin or to chymotrypsin-Sephadex by 3-phenylpropionic acid at pH 8.1 and 26°. TNS concentration, 3.5×10^{-4} M. Excitation wavelength, 360 nm. The fluorescence was recorded at 450 nm with an Aminco-Keirs spectrofluorometer. Curve I: free chymotrypsin. Curve II: chymotrypsin-Sephadex. Curve III: chymotrypsin-Sephadex heat treated at 60° for 15 min. The intensity values are arbitrarily adjusted to 100 for the intensity in the absence of 3-phenylpropionic acid.

extrapolated for very high concentrations of dye, I_{max}, is 80% higher than for the untreated enzyme. In both cases the bound dye could be eluted by buffer and a new titration performed with identical results.

The complex of chymotrypsin and TNS undergoes nearly complete quenching of the fluorescence of the dye upon binding of competitive inhibitors of the enzyme, such as D-tryptophan or 3-phenylpropionic acid (Figure 9, curve I) (McClure and Edelman, 1967). The fluorescence of TNS bound to chymotrypsin-Sephadex was also quenched upon the addition of 3-phenylpropionic acid. However, the intensity levelled off at 65% of its initial value at saturating concentrations of the inhibitor. When the intensity was plotted *vs.* the logarithm of the inhibitor concentration a typical titration curve was obtained corresponding to a dissociation constant of 6×10^{-3} M for the inhibitor (Figure 9, curve II). When the heat-treated chymotrypsin-Sephadex was titrated in the same way, quenching of the dye fluorescence was observed only at considerably higher concentrations of the inhibitor; it amounted to 15% at an inhibitor concentration of 10^{-1} M (Figure 9, curve III).

Discussion

The application of fluorometry as a tool for the study of conformational changes in proteins in solution has been extended in the present work to the investigation of proteins covalently bound to insoluble carriers. Most other physical-chemical methods in common use in the study of proteins in solution are impractical in the case of insolubilized proteins. This work illustrates how fluorescence measurements complement studies on enzymic activity of insolubilized enzymes and provide information not obtainable by activity measurements alone.

An inherent difficulty in the study of proteins bound to insoluble carriers by means of optical methods is the extensive scattering of light by the carriers. Fortunately for the fluorometric technique, the protein concentration in the protein-carrier conjugates studied is fairly high, being up to 1% protein/ml bed volume. The optical density per 1.0-mm pathlength at the protein absorption peak (280 nm) is thus about 2. Ninety per cent of the excitation light is absorbed within 0.5 mm of the front face of the sample (corresponding to about five layers of Sephadex spheres). The excitation beam is therefore predominantly absorbed rather than scattered. Under these conditions, the fluorescence light has of course to be collected from the front face.

The fluorometric cell used in this study was designed to fulfill the above requirement. Furthermore, since most of the insolubilized proteins are suitable column packing materials, the fluorometric cell was also designed to serve as a micro column. Thus many measurements can be performed on the sample, provided the conditions used do not irreversibly affect the protein. Differences in measurements due to differences in packing of the column are eliminated. Procedures equivalent to dialysis or ultrafiltration can conveniently be performed, with the resulting saving of time. Last but not least, very small quantities of materials are needed, the cell volume being about 100 μl, which requires about 1 mg of protein/study.

In the present study unpolarized light was used for the excitation of fluorescence. The emitted light was collected at 90° to the excitation beam. No attempt was made to study the polarization of the emitted light in view of uncertainties that may be introduced in the results by some scattering of the emitted light by the gel particles. To the extent that the measured changes in fluorescence intensity are contributed by changes in fluorescence polarization, they may still be used as a diagnostic tool for the detection of conformational changes in the bound proteins. As pointed out above (under Fluorescence Measurements in the Materials and Methods section), the changes in fluorescence intensity due to the highest possible changes in polarization in our measurements do not exceed 10%. In practice the changes in polarization expected to accompany changes in protein conformation are not expected to be extreme (see, for example, Weber and Young, 1964). The measured intensities at right angle to the excitation beam are thus an approximate measure of the total fluorescence intensity.

One should of course be aware of the limitations of the front-face fluorometric method. The optical density of the fluorophores has to be high at the wavelength of the excitation light. There is always some scattering of the excitation light; fluorophores with an emission maximum near the excitation light, *e.g.*, tyrosine, may therefore be difficult to detect without interference. In such cases, excitation light of shorter wavelength should be selected if possible. Chromophores present in the carrier which absorb light or fluoresce in the spectral region where the protein emits light may seriously interfere with the measurements. Similar interference may arise if the carrier absorbs the excitation light. Thus, proteins bound to carriers containing aromatic structures, azo groups, etc., are probably not amenable to fluorometric study.

Sephadex and cyanogen bromide activated Sephadex did not exhibit any fluorescence when illuminated with light of 280- or 360-nm wavelength. This result is to be expected in view of the chemical constitution of Sephadex and the fact that the cyanogen bromide activation process is not likely to introduce fluorescent groups into the gel. Sephadex and the similar polymer agarose are thus suitable carriers for fluorometric study of bound proteins.

The fluorescence spectrum of β-alanyltryptophan was the same in water and in 10% dextran solution, both in shape and in intensity. The highly hydrophilic polymer thus forms an

environment to the indole fluorophore which is identical with the aqueous environment as far as fluorescence properties are concerned. Similar results were obtained previously with sucrose (Steiner *et al.*, 1964). Furthermore, the fluorescence properties of β-alanyl-L-tryptophan-Sephadex were the same as those of the free peptide, both in water and in 8 M urea. These results are of importance, since they imply that changes in fluorescence of proteins bound to Sephadex or agarose should not be attributed to a change in the outside environment of the protein molecules. Such changes in protein fluorescence are rather indicative of conformational changes of the protein molecules.

This conclusion gains further support from the observation that TLCK-trypsin yielded fluorescence spectra of the same shape when measured in a fluorescence cell filled with water or in a fluorescence cell filled with Sephadex packing. The intensity of the emitted light was, however, lower in the latter case, which is probably due to partial exclusion of the protein from the gel and to attenuation of the excitation beam due to light scattering.

It may be noted that the fluorometric titration curve of β-alanyl-L-tryptophan-Sephadex is not identical with that of the free peptide in solution. It is known that the state of ionization of the amino group of the peptide has a profound effect on the fluorescence yield of the indole fluorophore (Edelhoch *et al.*, 1967). Indeed, the fluorometric titration of the free peptide shows a pK value of 9.35, that of the ionization of the amino group. The effect of the coupling reaction on amino groups has been studied (Axén *et al.*, 1970; Axen and Ernback, 1971; Porath and Fryklund, 1970); some of the amino groups were shown to change their basic properties by the coupling reaction. These changes are probably reflected in the modified fluorimetric titration curve of the bound peptide.

A striking parallelism was found between the effect of urea on the fluorescence properties of insolubilized trypsin and chymotrypsin and the effect of urea on the enzymic activity of the insolubilized enzymes. Chymotrypsin covalently bound to Sephadex or agarose and trypsin covalently bound to agarose completely lose their enzymic activity in 8 M urea (Gabel *et al.*, 1970; P. Vretblad and D. Gabel, unpublished observations). The fluorescence peak of these three enzyme derivatives shifts to the red by about 20 nm upon exposure to 8 M urea. A similar shift in the tryptophan fluorescence of trypsin and chymotrypsin in solution upon exposure to 8 M urea was observed before (Hopkins and Spikes, 1967, 1968), accompanying the loss in enzymic activity. In contradistinction to the above, trypsin covalently bound to Sephadex retains most of its initial enzymic activity when assayed in 8 M urea. Similarly, its fluorescence peak hardly shifts in wavelength or changes its intensity upon exposure to 8 M urea. It is thus evident that whenever the active site in the trypsin-agarose, chymotrypsin-Sephadex, and chymotrypsin-agarose conjugates is impaired by urea, additional changes occur in the conformation of the protein molecules profound enough to markedly affect the environment of the tryptophan residues. In the case of the trypsin-Sephadex conjugate the carrier stabilizes the molecular conformation of the protein against the effect of urea so that neither the active site of the enzyme nor the environment of the tryptophan residues is affected. There is other evidence that the carrier in the trypsin-Sephadex conjugate also stabilizes the protein against heat denaturation (D. Gabel, to be published).

Chymotrypsin covalently attached to Sephadex closely resembles chymotrypsin in solution also in the manner in which it binds the dye TNS. The resulting complex exhibits the same fluorescence in both cases (peak at 455 nm). Only a slight, possibly insignificant, difference was found in the dissociation constant of the protein–dye complex in the two cases (1.7×10^{-4} M for the insolubilized protein as compared to 2.0×10^{-4} M for chymotrypsin in water (McClure and Edelman, 1967)). It should be recalled that TNS does not bind at the active site of chymotrypsin and that substrate analogs such as D-tryptophan and 3-phenylpropionic acid do not compete with the dye bound to the protein. Upon binding, the substrate analogs quench, however, the fluorescence of the dye in the complex (McClure and Edelman, 1967). It is of interest that also in the case of the complex of TNS and chymotrypsin-Sephadex the addition of 3-phenylpropionic acid caused some quenching of the dye fluorescence. From the fluorometric titration curve a value of 6×10^{-3} M was calculated for the dissociation constant of the substrate analog bound to the insolubilized protein. This value should be compared with the corresponding value of 4.5×10^{-3} M (Kaufman and Neurath, 1949) or 4.2×10^{-3} M (McClure and Edelman, 1967), obtained for chymotrypsin in solution. The slight increase in the observed constant for the insolubilized enzyme might be due to the small amount of negative charge present in the activated gel (R. Axén and O. Carlson, to be published).

Although the binding strength of chymotrypsin-Sephadex toward TNS or toward the substrate analog 3-phenylpropionic acid is roughly the same as that of the enzyme in solution, an interesting difference between the two exists. The binding of the substrate analog to the TNS–protein complex in solution results in complete quenching of the fluorescence of the dye; the analogous binding in the case of the chymotrypsin-Sephadex conjugate resulted in 35% quenching only. An explanation for this discrepancy which is based on the assumption that 65% of the chymotrypsin in the Sephadex conjugate is inactive and thus does not bind the substrate analog is unacceptable. Thus, Axén *et al.* (1970) have demonstrated that the enzymic activity of chymotrypsin-Sephadex, extrapolated to high substrate concentration, *i.e.*, V_{max}, is the same as for chymotrypsin in solution. We are therefore led to the conclusion that while the Sephadex matrix does not appreciably modify the binding sites for TNS or substrate, it does interfere with the *interaction* between the sites, conceivably by limiting the flexibility of the covalently attached macromolecules of the enzyme.

The enzymic activity of chymotrypsin in solution and chymotrypsin bound to Sephadex at various temperatures is very similar (Figure 6, curve I). Both show maximum activity at 38°, which drops to zero at 55°. However, the stability of soluble and insolubilized chymotrypsin to heat inactivation is markedly different. (Heat inactivation was monitored by the residual enzymic activity, measured at 25°, after exposure of the enzyme to higher temperatures for a specified period of time.) Chymotrypsin in solution was relatively unstable at elevated temperatures and was nearly completely denatured at 50° for 5 min. This happens by autolysis, autopolymerization, and unimolecular inactivation processes (Kumar and Hein, 1970). Chymotrypsin bound to Sephadex behaved entirely differently. Only partial loss in activity was found to occur, even when kept at 60° for 15 min; using *N*-Ac-L-Tyr-OEt as substrate it was found that V_{max} dropped to 40% of its value for the insolubilized enzyme before heat treatment. This may be due to complete denaturation of 60% of the enzyme or to partial inactivation of all the enzyme molecules. An inflection point in the stability curve of chymotrypsin-Sephadex was observed at 47° (Figure 5, curve II). A similar behavior had been observed with chymotrypsin covalently

bound to carboxymethylcellulose (Surovtzev *et al.*, 1970). This is in accord with previous observations that proteolytic enzymes are protected from autodigestion and other intermolecular processes by binding to an insoluble polymer (*e.g.*, Bar-Eli and Katchalski, 1963; Levin *et al.*, 1964; Gabel and v. Hofsten, 1970; Goldstein *et al.*, 1970; Conte and Lehmann, 1971; Glassmeyer and Ogle, 1971).

The complementary fluorescence studies of chymotrypsin bound to Sephadex at various temperatures have revealed that the effect of temperature on the insolubilized chymotrypsin is more complex and more interesting than could be surmized from the enzymic activity measurements alone. On raising the temperature the emission peak shifts from 335 nm at room temperature to 352 nm at temperatures above 50°. In parallel to the stability curve as determined by enzymic activity (at 25°) an inflection point occurs at about 47°. However, upon cooling the chymotrypsin-Sephadex conjugate, the emission peak does not revert to its initial position; it stays instead at 352 nm. The heat-treated chymotrypsin-Sephadex thus differs significantly from the native enzyme in its fluorescence properties, though it exhibits a significant amount of enzymic activity. The red-shifted fluorescence indicates that the tryptophan residues in the heat-treated insolubilized enzyme are in a significantly more hydrophilic environment than in the native enzyme, which implies differences in conformation between the heat-treated bound enzyme and the native enzyme. It is pertinent to note that there is also a significant difference in fluorescence intensity between the heat-treated and native enzymes (Figure 7, lower part), which points to the same conclusion.

The heat-treated chymotrypsin-Sephadex conjugate is capable of binding TNS. The dissociation constant for this protein–dye complex was found to be 7×10^{-4} M from a plot of I *vs.* I/c_{total} according to eq 1, as compared to 1.7×10^{-4} M obtained before heat treatment. The binding site for TNS was thus partially impaired by the heat treatment. It is pertinent to note that if the protein preparation is not homogeneous with respect to the binding constant for the dye, a plot of I *vs.* I/c will deviate from a straight line. For example, curves III and IV in Figure 8 are calculated plots of I *vs.* I/c for a mixture of two species. The two species were assumed to be present in equal amounts. It was further assumed that one of the species had a dissociation constant for the protein–TNS complex of 2×10^{-4} M, and the other a dissociation constant of either 7×10^{-4} M (curve III) or 10×10^{-4} M (curve IV). The value of I_{max} for the first species was set to 55, the corresponding value for the second to 165, thus yielding a summed value of 220, which fits the experimentally observed I_{max}. The data presented in Figure 8 (curve II) thus suggest that the heat-treated chymotrypsin-Sephadex conjugate is homogeneous as judged by the binding of TNS.

The heat-treated chymotrypsin-Sephadex also differed from the untreated conjugate in another respect. When its TNS complex was titrated with 3-phenylpropionic acid the fluorescence was not quenched at substrate analog concentrations below 10^{-2} M (see Figure 9, curve III). At a concentration of 10^{-1} M of the analog the fluorescence dropped to 85% of its initial value. A complete titration could not be performed due to the limited solubility of the substrate analog. The heat treatment either weakened the binding between the enzyme and 3-phenylpropionic acid or destroyed the interaction between the binding site for the substrate analog and that for TNS.

The above findings indicate that chymotrypsin-Sephadex heat treated for 15 min at 60° has some conformational properties which are quite distinct from those of chymotrypsin in solution, though it possesses appreciable catalytic activity.

As shown in this work, trypsin and chymotrypsin bound to cyanogen bromide activated Sephadex differ from the untreated enzymes in several respects. While their catalytic characteristics are most probably retained during the coupling process, factors known to cause conformational changes in proteins seem to affect the bound and the free enzyme differently. In the cases described the bound enzymes are more resistant than the corresponding native enzymes. It should be borne in mind, however, that labilization of enzymes as a result of covalent binding to different carriers has been recorded in several cases (Cresswell and Sanderson, 1970; Erlanger *et al.*, 1970; Goldman *et al.*, 1971).

The fluorescence method presented here enables the detection of conformational changes in proteins bound to insoluble carriers. Such changes which might be caused by the binding to the carrier, by solvents, temperature, substrates, inhibitors, activators, or haptens, are difficult to detect in multiphase systems by other physical–chemical techniques. The fluorescence method should be also useful in detecting conformational changes in membrane-bound enzymes, and enzyme aggregates.

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Enzymatic Properties of Uroporphyrinogen III Cosynthetase*

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ABSTRACT: Uroporphyrinogen III cosynthetase is inactivated during the formation of uroporphyrinogen III catalyzed by uroporphyrinogen I synthetase. However, cosynthetase has the physical and chemical properties of a protein, and is active in catalytic concentrations. This indicates that cosynthetase is an enzyme which is inactivated during the course of the reaction which it catalyzes. Although under standard conditions the amount of uroporphyrinogen III formed is proportional to the amount of cosynthetase inactivated, the proportionality can be altered by changing the amount of synthetase present. Hence, the inactivation of cosynthetase is probably not an essential part of its catalytic activity. Cosynthetase from mouse spleen can be purified 18-fold with a 5% yield by ammonium sulfate fractionation and chromatography on Bio-Rex 70. It is inhibited about 80% by 2×10^{-5} M concentrations of uroporphyrins and coproporphyrins, or their corresponding porphyrinogens. Its pH optimum is about 7.7–7.9, the same as that for mouse spleen uroporphyrinogen I synthetase. At higher pH values, around 9.0, cosynthetase stimulates the activity of synthetase, and this stimulation can be used as a rapid assay for purified cosynthetase.

Two separate fractions are required for the formation of uroporphyrinogen III by extracts of plant or animal tissues (Bogorad, 1962; Levin and Coleman, 1967). One of these fractions is the enzyme uroporphyrinogen I synthetase, which catalyzes the formation of 1 mole of uroporphyrinogen I from 4 moles of porphobilinogen. Uroporphyrinogen III is formed instead of uroporphyrinogen I when synthetase acts upon porphobilinogen in the presence of the second fraction, called uroporphyrinogen III cosynthetase. Cosynthetase alone does not catalyze the disappearance of porphobilinogen, and it is not an isomerase which catalyzes interconversion of the uroporphyrinogen isomers.

Studies on uroporphyrinogen I synthetase and uroporphyrinogen III cosynthetase separated from erythropoietic mouse spleen revealed that cosynthetase activity is destroyed during the formation of uroporphyrinogen III. Under certain reaction conditions, the amount of uroporphyrinogen III formed is proportional to the amount of cosynthetase disappearing (Levin, 1968a). This relationship raised the possibility that cosynthetase functions as a nonrecycling cofactor rather than as a catalyst. However, a number of enzymes are known to be inactivated during the course of the reactions which they catalyze (McLemore and Metzler, 1968; Powers and Dawson, 1944; Hager *et al.*, 1957; Zervos *et al.*, 1971), and the question of the enzymatic nature of uroporphyrinogen III cosynthetase has remained unsettled.

The present report shows that uroporphyrinogen III cosynthetase is a proteinaceous material which is active in such low concentrations that it must be assumed to be participating in the formation of a large number of equivalents of uroporphyrinogen III before being inactivated—that is, to be functioning catalytically, as an enzyme. The proportionality between cosynthetase inactivation and uroporphyrinogen III formation can be altered by changing the incubation conditions, which suggests that the reaction which inactivates cosynthetase is not an essential part of the formation of uroporphyrinogen III. A modified assay for cosynthetase, which gives absolute values for the amount of uroporphyrinogen III formed rather than the relative values obtained previously,

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